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Biphasic Effect of Melanocortin Agonists on Metabolic Rate and Body Temperature

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SUMMARY

The melanocortin system regulates metabolic homeostasis and inflammation. Melanocortin agonists have contradictorily been reported to both increase and decrease metabolic rate and body temperature. We find two distinct physiologic responses occurring at similar doses. Intraperitoneal administration of the nonselective melanocortin agonist MTII causes a melanocortin-4 receptor (Mc4r)-mediated hypermetabolism/hyperthermia. This is preceded by a profound, transient hypometabolism/hypothermia that is preserved in mice lacking any one of Mc1r, Mc3r, Mc4r, or Mc5r. Three other melanocortin agonists also caused hypothermia, which is actively achieved via seeking a cool environment, vasodilation, and inhibition of brown adipose tissue thermogenesis. These results suggest that the hypometabolic/hypothermic effect of MTII is not due to a failure of thermoregulation. The hypometabolism/hypothermia was prevented by dopamine antagonists, and MTII selectively activated arcuate nucleus dopaminergic neurons, suggesting that these neurons may contribute to the hypometabolism/hypothermia. We propose that the hypometabolism/hypothermia is a regulated response, potentially beneficial during extreme physiologic stress.

INTRODUCTION

Tight and flexible control of energy homeostasis is essential for survival, of both the individual and the species. The evolving epidemics of obesity and type 2 diabetes are likely the result of environmental changes interacting with genetic variation, causing an imbalance between energy assimilation and energy expenditure. A more complete understanding of the physiologic control mechanisms should lead to improved therapies for obesity and diabetes.

The melanocortin system helps control energy homeostasis (Cone, 2005, 2006; Tao, 2010). Of the five melanocortin receptors, Mc4r has received the most attention: mice become obese when they express agouti, a protein inhibitor of Mc4r (Yen et al., 1994), when Mc4r function is disrupted (Huszar et al., 1997), or when the endogenous ligands (e.g., α-melanocyte-stimulating hormone, α-MSH) derived from pro-opiomelanocortin are lost (Yaswen et al., 1999). Similarly, human obesity can be caused by reduced MC4R function (Vaisse et al., 1998; Yeo et al., 1998) or by loss of agonist ligand (Krude et al., 1998). *MC4R* mutations account for ~1%–5% of extreme human obesity (Alharbi et al., 2007; Stutzmann et al., 2008), and common variants near *MC4R* are also associated with obesity (Loos et al., 2008). MC4R agonists are being considered for the treatment of obesity, as they reduce food intake, increase metabolic rate, and increase insulin sensitivity (e.g., Kievit et al., 2013). However, MC4R agonists also elevate blood pressure (Greenfield et al., 2009; Silva et al., 2006) and increase erectile activity (Van der Ploeg et al., 2002).

α-MSH also binds to and activates MC1R, MC3R, and MC5R, but not MC2R. MC1R agonism causes darkening of skin and hair (Robbins et al., 1993) and reduces inflammation (Leoni et al., 2010; Li and Taylor, 2008), while loss of MC1R function reduces sensitivity to certain painful stimuli (Mogil et al., 2003, 2005). MC3R contributes to the control of energy homeostasis (e.g., null mice are mildly obese; Butler et al., 2000; Chen et al., 2000a), natriuresis (Ni et al., 2003), and inflammation, acting at least partially on macrophages (Getting et al., 2008). Genetic variation in MC3R may contribute to human obesity (Feng et al., 2005; Renquist et al., 2011). MC5R regulates exocrine secretion (Chen et al., 1997) and inflammation (Lee and Taylor, 2013).

With the focus on melanocortins in obesity, an older, sometimes contradictory, literature investigating stress, inflammation, and core body temperature (Tb) has received less attention. Lipton and Glyn (1980) reported that α-MSH reduced rabbit rectal temperature and found that low doses of α-MSH prevented lipopolysaccharide-induced fever (Catania and Lipton, 1993; Murphy et al., 1983). In contrast, α-MSH increased Tb in rats (Raible and Knickerbocker, 1993; Resch and Simpson, 1991). Much of this Tb work was performed using nonselective ligands, such as α-MSH and MTII (Haskell-Luevano et al., 1997), before the identification of all five melanocortin receptors. MC4R-selective

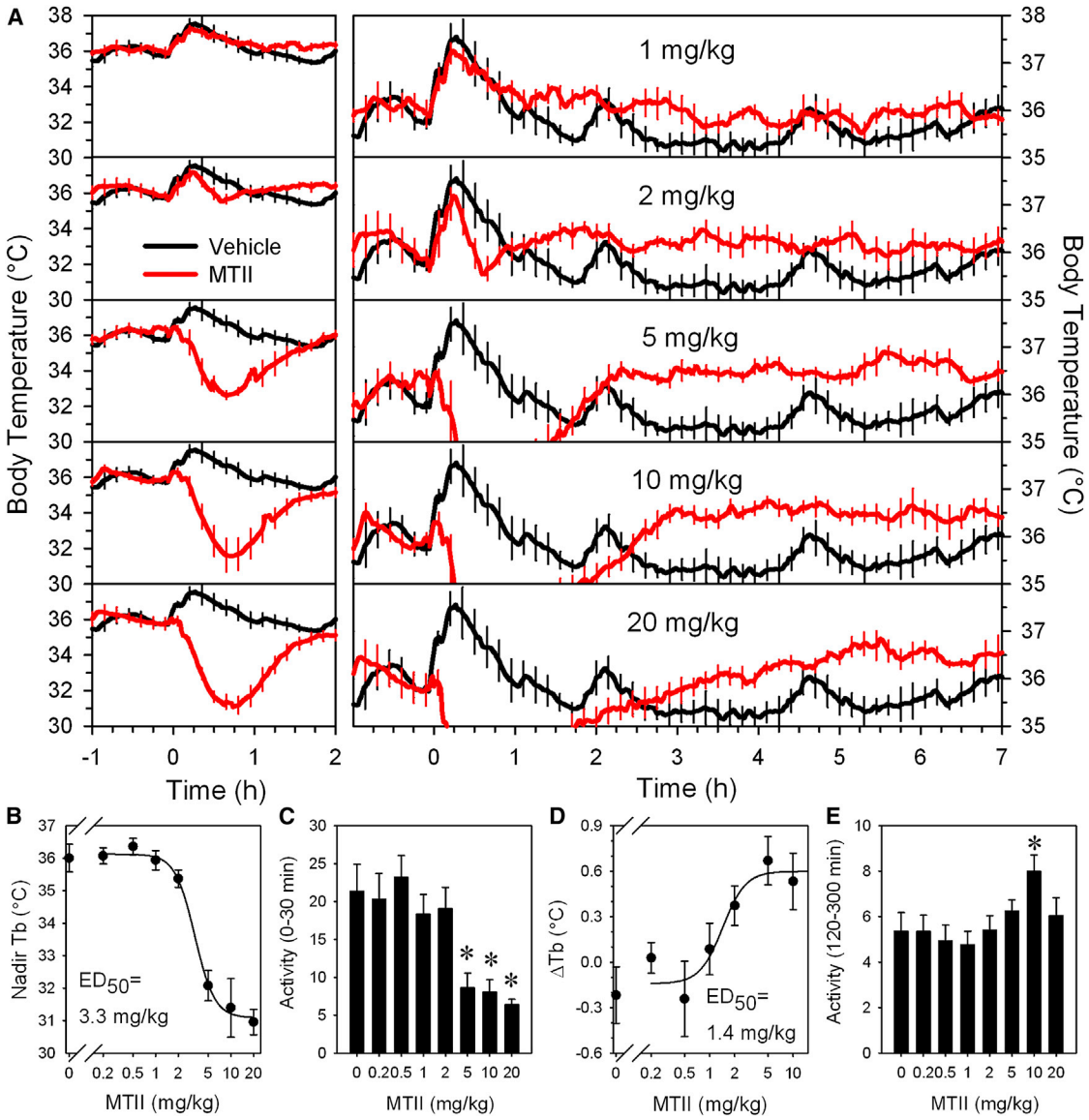


Figure 1. MTII Tb Dose Response in Lean C57BL/6J Mice

(A) Tb response to the indicated MTII dose (red) or vehicle (black, repeated in each panel) injected into chow-fed mice (mean of n = 6/group, body weight 27.1 ± 0.3 g) studied at 21°C–22°C. Left and right panels show the same data with different Tb axis scales to allow visualization of both effects. Every fifth SEM is shown for visual clarity.

(B–E) Dose response curves for the nadir in Tb (B), physical activity (mean, 0–30 min) (C), peak increase in Tb (difference between the mean of −150 to −30 min and the mean of 120–300 min) (D), and physical activity (mean, 120 to 300 min) (E) from the analyses of the data shown in (A). Activity is in arbitrary units via Mini Mitter. *$p < 0.05$ versus pooled vehicle, 0.2, and 0.5 mg/kg MTII data. Data are mean ± SEM.

agonists can both reduce and increase Tb, although a non-MC4R contribution is also suspected (Metzger et al., 2010; Nicholson et al., 2007; Sinha et al., 2003, 2004).

The mouse, due to its small body size, exhibits amplified changes in Tb and responses to manipulation of environmental temperature (Gordon, 1993, 2012). The large Tb changes and available genetic variants make the mouse an ideal system for studying the thermal biology of melanocortins. While melanocortin agonists typically increase metabolic rate (Chen et al., 2000b), there is also a report of a metabolic rate reduction (Wisse et al., 2006). Here, we study the effects of melanocortin agonism on metabolic rate and Tb, finding divergent effects with importance for both energy homeostasis and the control of inflammation.

RESULTS

Biphasic Effect of Treatment with MTII on Energy Expenditure and Tb

The nonselective melanocortin agonist, MTII, has a biphasic effect, first decreasing and then increasing Tb in chow-fed C57BL/6J mice (Figure 1A). These effects are in addition to the

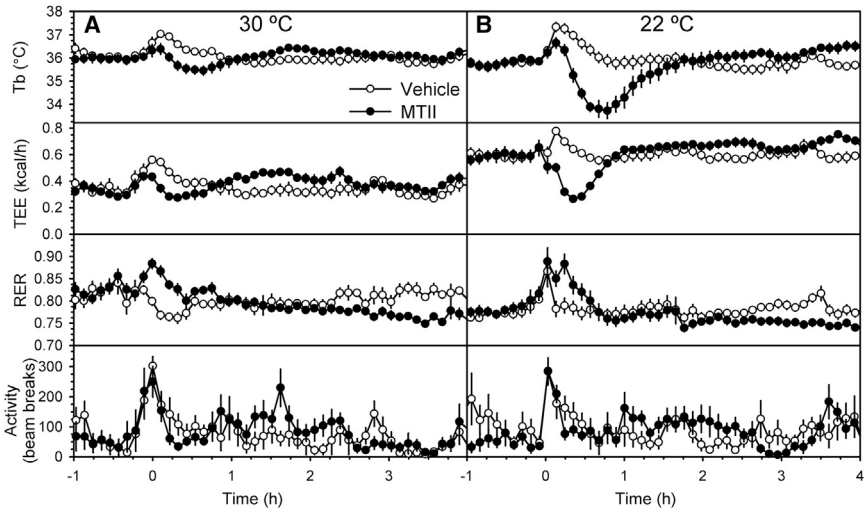


Figure 2. Thermogenic Effects of MTII Treatment in Diet-Induced Obese C57BL/6J Mice Housed at Thermoneutrality or Room Temperature

(A) Tb, TEE, RER, and activity in DIO mice (mean body weight, 45.6 g) treated with MTII (black circles) or vehicle (white circles) in a randomized crossover design studied at 30°C (n = 6/group).

(B) Tb, TEE, RER, and activity in the same mice studied at 22°C (n = 9/group). Data are mean ± SEM.

stress of handling, which initially increases both Tb and physical activity in mice treated with either vehicle or MTII. The effective dose 50 (ED_{50}) for the hypothermic effect of MTII was 3.3 ± 0.5 mg/kg (Figure 1B). The hypothermia effect reached a plateau at the highest doses (5, 10, and 20 mg/kg), with these doses exhibiting a similar nadir Tb, time to nadir Tb (35 ± 1, 38 ± 3, and 37 ± 3 min), and maximum cooling rate (−1.33 ± 0.08, −1.37 ± 0.08, and −1.50 ± 0.12°C/5 min at ~8–14 min after dosing). During hypometabolism/hypothermia, physical activity was reduced (Figure 1C). The late Tb increase produced by MTII had an ED_{50} of 1.4 ± 0.5 mg/kg (Figure 1D) and was sometimes accompanied by slight increases in physical activity (Figure 1E).

MTII's hypothermic and hyperthermic effects in diet-induced obese (DIO) mice (46.1 ± 0.8 g) were similar to those in the chow-fed mice. The hypothermia ED_{50} was 3.3 ± 0.6 mg/kg, and the hyperthermia ED_{50} was 2.0 ± 0.8 mg/kg. The nadir Tb was 32.7 ± 0.5, 31.7 ± 0.3, and 31.4 ± 0.8°C at 5, 10, and 20 mg/kg, respectively, occurring at 41 ± 3, 43 ± 3, and 42 ± 4 min.

The effects of pharmacologic agents on metabolic rate are typically studied at thermoneutrality to neutralize the contribution of facultative thermogenesis. In DIO mice, the MTII-induced hypothermia at thermoneutrality (30°C) was truncated compared to the effect at 22°C (Figure 2). Like its effect on Tb, MTII decreased and then increased total energy expenditure (TEE), while it had the opposite effect (increase then decrease) on respiratory exchange ratio (RER, indicating a decrease then increase in fractional fat oxidation). In DIO mice at 22°C, the Tb reduction was ~4°C, and the TEE reduction was ~50% (Figure 2B). The nadir in TEE precedes that for Tb (at 23.8 ± 1.6 min for TEE versus 39.9 ± 2.8 min for Tb; $p < 0.001$). The late increase in Tb and TEE and decrease in RER were seen at both 22°C and 30°C.

Interestingly, hypothermia was not observed with every MTII treatment—it was not seen after 9% of 155 injections in 65 mice. All 11 nonresponding mice that were tested more than once did show hypothermia with previous or subsequent MTII treatment. The hypothermic response to MTII was preserved in the setting of a cage switch (Lee et al., 2004) (Figure S1, available online). This suggests that stress, as caused by cage switch, does not prevent the hypothermic response.

Hypothermia Generation Is an Active Process with Reduced Heat Generation and Ongoing Heat Loss

The physiological basis for the hypometabolism/hypothermia was investigated next. Brown adipose tissue (BAT) temperature is ~1°C higher than Tb, but at 20 min after MTII dosing, this BAT-Tb difference was abolished. By 50 and 180 min after dosing, the BAT-Tb differential was reestablished (Figures 3A–3C). The mice were cooler over their full surface area; no preferred sites for heat loss were detected using infrared thermography (Figure 3D). These data demonstrate that BAT inactivation contributes to the hypothermic effect of MTII and suggest that BAT reactivation contributes to rewarming. However, rewarming occurred normally in *Ucp1*$^{-/-}$ mice (Figure 3E), suggesting that other heat-generating mechanisms can substitute for the lack of BAT functionality.

Next, we compared the rate of MTII-induced Tb reduction to the rate of heat loss after death (Figure 3F). The times for Tb to fall from 35.0°C to 33.5°C were 8.5 ± 1.0 and 7.3 ± 0.2 min in two euthanasia groups, compared with 9.0 ± 1.3 min in MTII-treated mice (not statistically different). Thus, the rate of Tb reduction by MTII approaches that seen with cessation of metabolism.

When mice were allowed to choose their environment after MTII administration, the preferred environmental temperature was ~5°C cooler than after treatment with vehicle, and the duration of the cool preference was similar to the duration of the hypothermia (Figure 3G). After moving to the cool region, the mice were largely immobile. No shivering was observed. Taken together, these data suggest that the mouse uses all available mechanisms in order to achieve profound hypometabolism/hypothermia.

Hypothermic Effect Attenuates with Repeated Dosing of MTII

When MTII was administered daily for 5 days, the late increase in Tb showed little or no attenuation, while the hypothermic effect was greatly attenuated by the second dose and not detectable subsequently (data not shown). The kinetics of the attenuation were explored by injecting two doses of MTII 2 hr apart. There was no hypothermia with the second MTII dose (Figure S2). This suggests that MTII hypometabolism/hypothermia is a time-limited response followed by an extended refractory period.

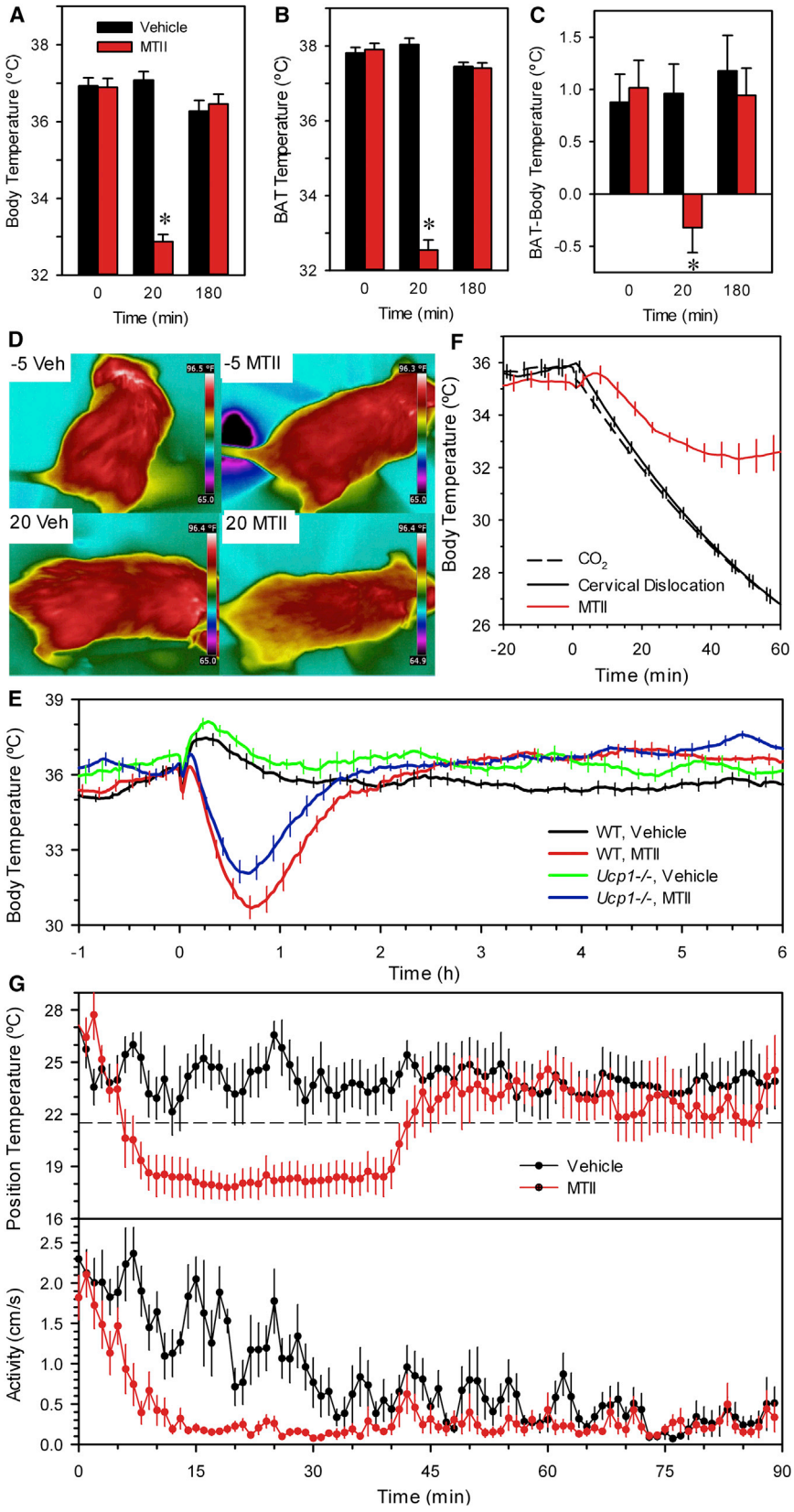


Figure 3. Physiology of the Hypothermic Response to MTII

(A–C) In chow-fed C57BL/6J mice after MTII (red) or vehicle (black) treatment, we show Tb (A), interscapular BAT temperature (B), and the differential between BAT temperature and Tb (C). Temperatures were measured at the indicated times after dosing. Data are mean ± SEM; n = 6/group; *p < 0.05 versus vehicle (mean weight 27.5 g).

(D) Infrared images of chow-fed mice taken 5 min before and 20 min after vehicle or MTII treatment using a FLIR T400 camera and analyzed using FLIR QuickReport 1.2 SP2 for measurement of temperatures within images.

(E) Response of *Ucp1*$^{-/-}$ mice to MTII at 22°C (body weight 26.1 ± 1.6 g; n = 8–10/group; crossover design, 8 male and 2 female). Every tenth SEM is shown for visual clarity.

(F) Rate of heat loss in DIO mice. Mice were euthanized by carbon dioxide (black dashed line) or cervical dislocation (black solid line) at time 0. As a concurrent control, live mice were administered MTII (red). Ambient temperature was 21.6°C (data are mean ± SEM; n = 6–7/group; body weight 52.1 ± 1.3 g). For visual clarity, every fifth SEM is graphed.

(G) Choice of environmental temperature. Mice on a chow diet were treated with MTII (red) or vehicle (black) and immediately placed in a thermal gradient, with position monitored by video (n = 8, crossover design). Ambient temperature was 21.6°C (dashed line).

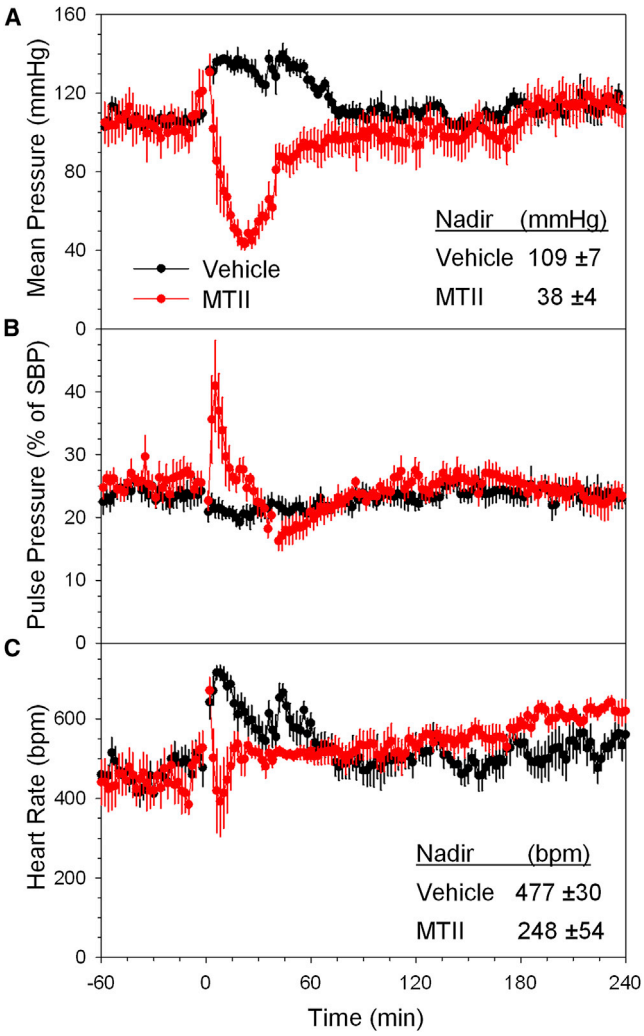


Figure 4. MTII Effect on Blood Pressure and Heart Rate

(A–C) We measured mean arterial pressure (A), pulse pressure (as a percent of systolic blood pressure) (B), and heart rate (C) in ambulating telemetered mice at 22°C treated with vehicle or MTII in a crossover design; n = 5–6/group. Tb was measured just prior to MTII injection (35.5°C ± 0.3°C) and again at 40 min (vehicle, 36.1°C ± 0.3°C; MTII, 31.1°C ± 0.3°C). Data are mean ± SEM.

Hypotension Accompanies MTII Hypometabolism/Hypothermia

Melanocortin activation causes an increase in blood pressure (Greenfield et al., 2009; Hall et al., 2010; Sohn et al., 2013). However, at early times after MTII dosing we observed profound hypotension, with a nadir arterial pressure of 109 ± 7 millimeters of mercury (mmHg) in the vehicle group versus 38 ± 4 mmHg after MTII ($p < 0.001$) (Figure 4A). Similar nadir changes were observed in the systolic (127 ± 6 versus 48 ± 11 mmHg; $p < 0.001$) and diastolic (99 ± 5 versus 32 ± 8 mmHg; $p < 0.001$) blood pressure. The pulse pressure increased, suggesting that vasodilation contributes to the hypotension (Figure 4B). The blood pressure nadir was at 28 min (median), similar in time course and duration to the drop in TEE. MTII treatment also decreased heart rate, but this effect was smaller, more variable, and of shorter duration than the effect on blood pressure (Figure 4C). Interestingly, the MTII hypotension was not accompanied by tachycardia at any time; the heart rate actually remained below that of the vehicle-treated controls, which exhibited the expected increase due to handling stress. At later times after MTII dosing, there was a suggestion of an increase in heart rate but no clear increase in blood pressure.

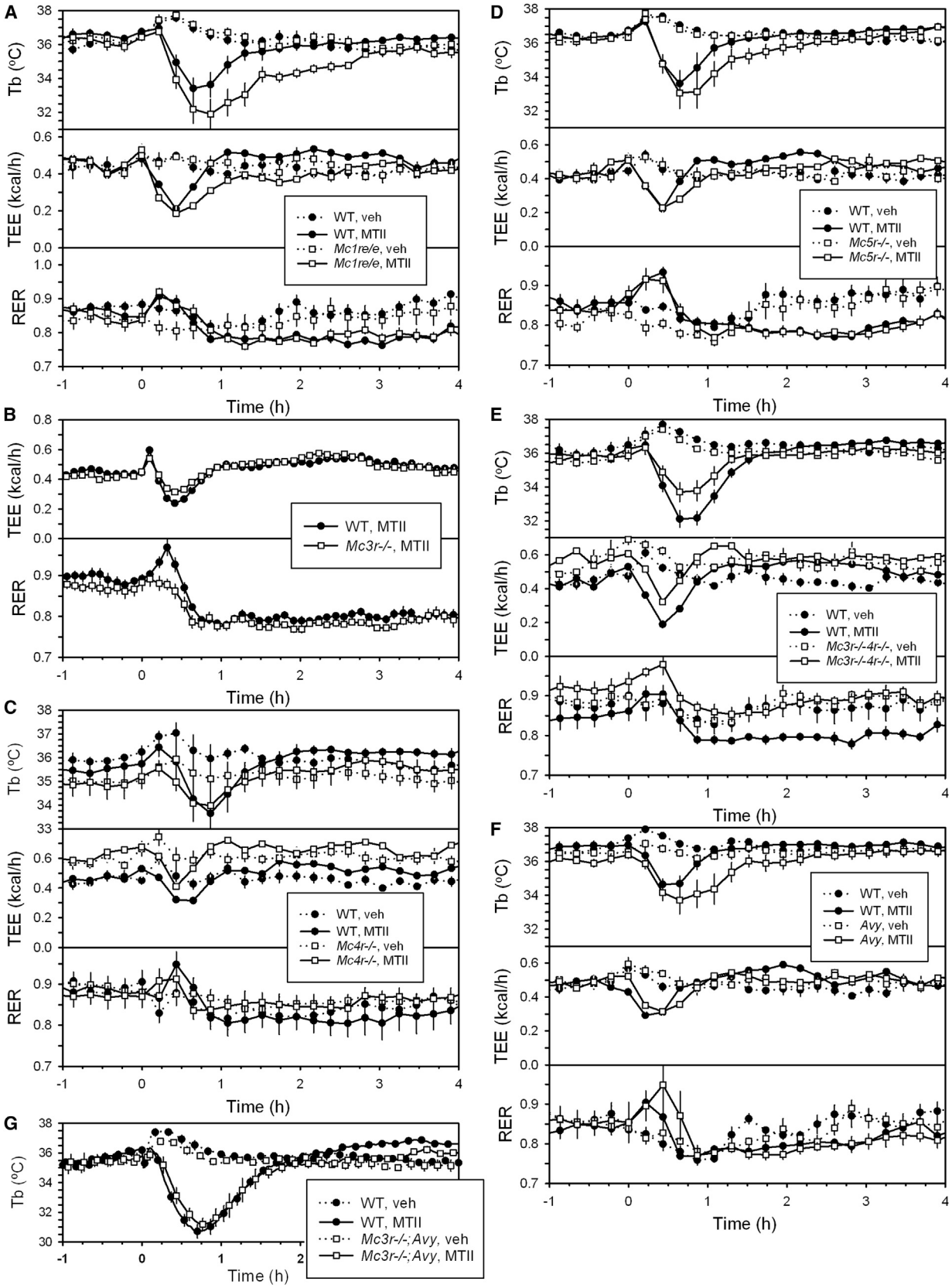
MTII Hypometabolism and Hypermetabolism in Melanocortin Mutant Mice

Since MTII binds four melanocortin receptors (MC1R, MC4R > MC3R > MC5R; Haskell-Luevano et al., 1997; see also Conde-Frieboes et al., 2012), we examined which receptors mediate the TEE and Tb effects in chow-fed mice. An initial hypometabolism/hypothermia was observed in $Mc1r^{e/e}$, $Mc3r^{-/-}$, $Mc4r^{-/-}$, and $Mc5r^{-/-}$ mice (Figures 5A–5D). It was also observed in $Mc3r^{-/-}$;$Mc4r^{-/-}$ double null mice, $A^{vy/+}$ mice ($A^{vy/+}$ mice have reduced Mc1r and Mc4r signaling), and $Mc3r^{-/-}$;$A^{vy/+}$ mice (Figures 5E–5G). These results indicate that neither Mc1r, Mc3r, Mc4r, nor Mc5r is individually required for the initial hypometabolic/hypothermic response. The late increase in TEE and reduction in RER (indicating increased fat oxidation) were lost in the $Mc4r^{-/-}$ and $Mc3r^{-/-}$;$Mc4r^{-/-}$ mice and retained in $Mc1r^{e/e}$, $Mc3r^{-/-}$, and $Mc5r^{-/-}$ mice, demonstrating that these late effects are dependent on Mc4r. These data demonstrate that different receptors control the hypometabolism/hypothermia versus the hypermetabolism/hyperthermia.

To further explore receptor specificity, we measured the effect of a MC1R agonist, BMS-470539 (Kang et al., 2006). While BMS-470539 at 30 mg/kg i.p. caused hypothermia in wild-type mice, hypothermia also occurred in $Mc1r^{e/e}$ mice (Figures S3A and S3B), indicating that this BMS-470539 effect does not require MC1R. We found that the small molecule MC4R agonist compound 2B (Guo et al., 2008) caused hypothermia and hypometabolism in both wild-type and $Mc4r^{-/-}$ mice (Figures S3C and S3D), indicating that hypothermia/hypometabolism with 40 mg/kg intraperitoneal (i.p.) compound 2B does not require MC4R. Another peptide melanocortin agonist, NDP-MSH ([Nle^4,D-Phe^7]-α-MSH) (Haskell-Luevano et al., 1997), also caused hypothermia with doses at the high end of those reported (Hoggard et al., 2004), and the hypothermia also occurred in $Mc3r^{-/-}$;$A^{vy/+}$ mice (Figure S3E).

Dopamine Receptor Antagonists Block MTII Hypometabolism/Hypothermia

To investigate possible mechanisms by which MTII causes hypothermia, we selectively blocked neurotransmitters that can cause hypothermia. Specifically, naloxone (10 mg/kg i.p.) blocks mu/kappa opioid receptor-mediated hypothermia (Baker and Meert, 2002), naltrindole (5 mg/kg i.p.) blocks delta opioid receptor-mediated hypothermia (Rawls and Cowan, 2006), WAY100635 (1 mg/kg subcutaneously) blocks serotonin 5-HT1A receptor-mediated hypothermia (Cryan et al., 2000; Rawls and Cowan, 2006), and AM251 (10 mg/kg i.p.) blocks cannabinoid-1 receptor-mediated hypothermia (McMahon and Koek, 2007). Each inhibitor was tested by itself and had no effect on Tb in the first hour after dosing. When dosed prior to MTII, none of the inhibitors ameliorated MTII hypothermia (data not shown). These data indicate that MTII hypothermia does not



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require signaling via opioid (mu, kappa, or delta), serotonin 5-HT1A, or cannabinoid-1 receptors.

Brain-penetrant dopamine agonists cause hypothermia via both D1-like and D2-like receptors (Carboni et al., 1986; Nunes et al., 1991), referred to hereafter as D1 and D2. Individually, a D1 antagonist (SCH23390, 2 mg/kg i.p.) or a D2 antagonist (sulpiride, 30 mg/kg i.p.) partially inhibited MTII hypothermia, while the combined inhibitors completely blocked MTII hypothermia (Figures 6A and 6B). D1/D2 combined inhibition also inhibited MTII-induced hypometabolism (Figures 6E–6H). MTII is not acting directly on either D1 or D2 receptors, as MTII does not bind with high affinity to these receptors (Figure S4). Physical activity was not affected by D2 antagonism and only modestly inhibited by D1 antagonism. However, activity was greatly inhibited by combined D1/D2 inhibition, irrespective of MTII treatment (Figures 6C and 6D). Decreased physical activity typically decreases Tb; thus, the prevention of MTII hypothermia by D1/D2 inhibition is in the opposite direction of that expected for an activity effect. The later decrease in RER caused by MTII is not inhibited by dopamine antagonists. Taken together, these data demonstrate that the hypothermia caused by MTII is reversed by D1/D2 blockers and is largely independent of physical activity. In contrast, combined D1/D2 inhibition did not ablate the hypotensive effect of MTII, despite slight increases in mean arterial pressure and reduction in pulse pressure (Figures 6I–6K). These results mechanistically distinguish the hypometabolism/hypothermia from the hypotension.

The hypothermic effect of the D2 agonist, quinpirole (1 mg/kg i.p.), attenuates with daily dosing (Buck et al., 2000). However, when given 2 hr apart, quinpirole-induced hypothermia did not attenuate, and there was no cross-attenuation of MTII and quinpirole (Figure 6L). Thus, attenuation of MTII hypometabolism/hypothermia does not appear to be a direct D2 effect.

MTII Selectively Activates Dopaminergic Neurons in the Arcuate Nucleus

The dopamine antagonist data suggest that MTII acts via dopaminergic neurons to cause hypothermia/hypometabolism. To identify such neurons, we looked in brain regions implicated in the regulation of Tb and EE for dopaminergic (tyrosine hydroxylase [TH]-immunoreactive) neurons activated (Fos-immunoreactive) at 1 hr after treatment with MTII. Of seven candidate regions, only the arcuate nucleus showed increased Fos staining after MTII treatment in TH-immunoreactive neurons (Figure 7). Since melanocortins can act via arcuate dopaminergic neurons to suppress prolactin secretion (Dutia et al., 2012), we measured Fos activation in *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ mice, which presumably lack this function. In *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ mice treated with MTII, the percentage of TH-immunoreactive neurons that stained for Fos, while reduced compared to wild-type mice, was significantly greater than that of vehicle-treated *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ mice (Figure 7). These data suggest that arcuate dopaminergic neurons may mediate the MTII-induced hypothermia.

DISCUSSION

The current studies aimed to unravel the contradictory observations that melanocortin agonists can both increase and decrease both metabolic rate and Tb. We found that MTII, the most commonly used melanocortin agonist, causes hypermetabolism/hyperthermia in mice, which is preceded by a transient hypometabolism/hypothermia/hypotension, with both effects occurring at similar doses. Immediately after MTII administration, there are dramatic reductions in metabolic rate (by ~50%), Tb (by ~4°C), and blood pressure (by ~65%) in awake mice at room temperature. Characteristics of the hypometabolic/hypothermic state include turning off BAT thermogenesis, rapid heat loss, active seeking of a cool environment, reduced physical activity, and no compensatory shivering. The TEE nadir at ~24 min precedes the Tb nadir at ~40 min, suggesting that hypometabolism drives the hypothermia. Thus, the hypometabolism is not simply a consequence of the reduced temperature per se causing the reduction in metabolic rate processes (known as a "Q_{10}" or temperature coefficient effect). While melanocortins cause heat loss via the tail in rat (Sinha et al., 2003) and ears in rabbit (Lipton et al., 1981), heat loss in the mouse appears generalized. Indeed, the hypotension and increased pulse pressure suggest that vasodilation is facilitating the heat loss. It is notable that the hypotension is not accompanied by a compensatory tachycardia, as occurs with some causes of hypotension, such as hypovolemia. The inactive BAT, hypotension, and lack of tachycardia are all evidence of reduced sympathetic tone. These observations demonstrate that reaching the hypothermic state is an active, coordinately regulated process, with reduced heat generation and without induction of heat-conserving mechanisms.

The hypometabolic/hypothermic response to MTII is preserved in mice lacking the function of any one of Mc1r, Mc3r, Mc4r, or Mc5r. Three other melanocortin ligands (NDP-MSH, compound 2B, and BMS-470539) also caused hypothermia, and these responses were also preserved in various melanocortin mutant mice, despite using doses previously believed to be selective. Thus, the hypometabolic/hypothermic response to MTII and other ligands appears to be mediated redundantly via melanocortin and/or other unidentified receptors. It is notable that the MTII ED_{50}s for both the hypothermic and hyperthermic responses are comparable and smaller than the 4–20 mg/kg doses typically used (e.g., Balthasar et al., 2005; Chen et al., 2000b; Chen et al., 2009).

Figure 5. Tb, TEE, and RER Response of Melanocortin Receptor Mutant Mice to MTII

(A) *Mc1r*$^{e/e}$ (21.6 ± 0.3 g) and wild-type (23.3 ± 0.8 g) male mice (n = 6/group).
(B) *Mc3r*$^{-/-}$ (27.3 ± 0.6 g) and wild-type (27.0 ± 0.5 g) male mice (n = 12/group).
(C) *Mc4r*$^{-/-}$ (54.1 ± 1.0 g) and wild-type (29.3 ± 0.6 g) male and female mice (n = 4–6/group).
(D) *Mc5r*$^{-/-}$ (23.3 ± 0.6 g) and wild-type (22.3 ± 0.6 g) male mice (n = 6/group).
(E) *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ (42.7 ± 1.6 g) and wild-type (25.6 ± 0.8 g) male and female mice (n = 6/ group).
(F) *A*^{vy}/+ (30.3 ± 1.5 g) and wild-type (22.5 ± 0.6 g) female mice (n = 5/group).
(G) *Mc3r*$^{-/-}$; *A*^{vy}/+ (37.6 ± 2.0 g, n = 5) and wild-type (28.5 ± 0.5 g, n = 9) male mice, crossover design. Data are mean ± SEM.

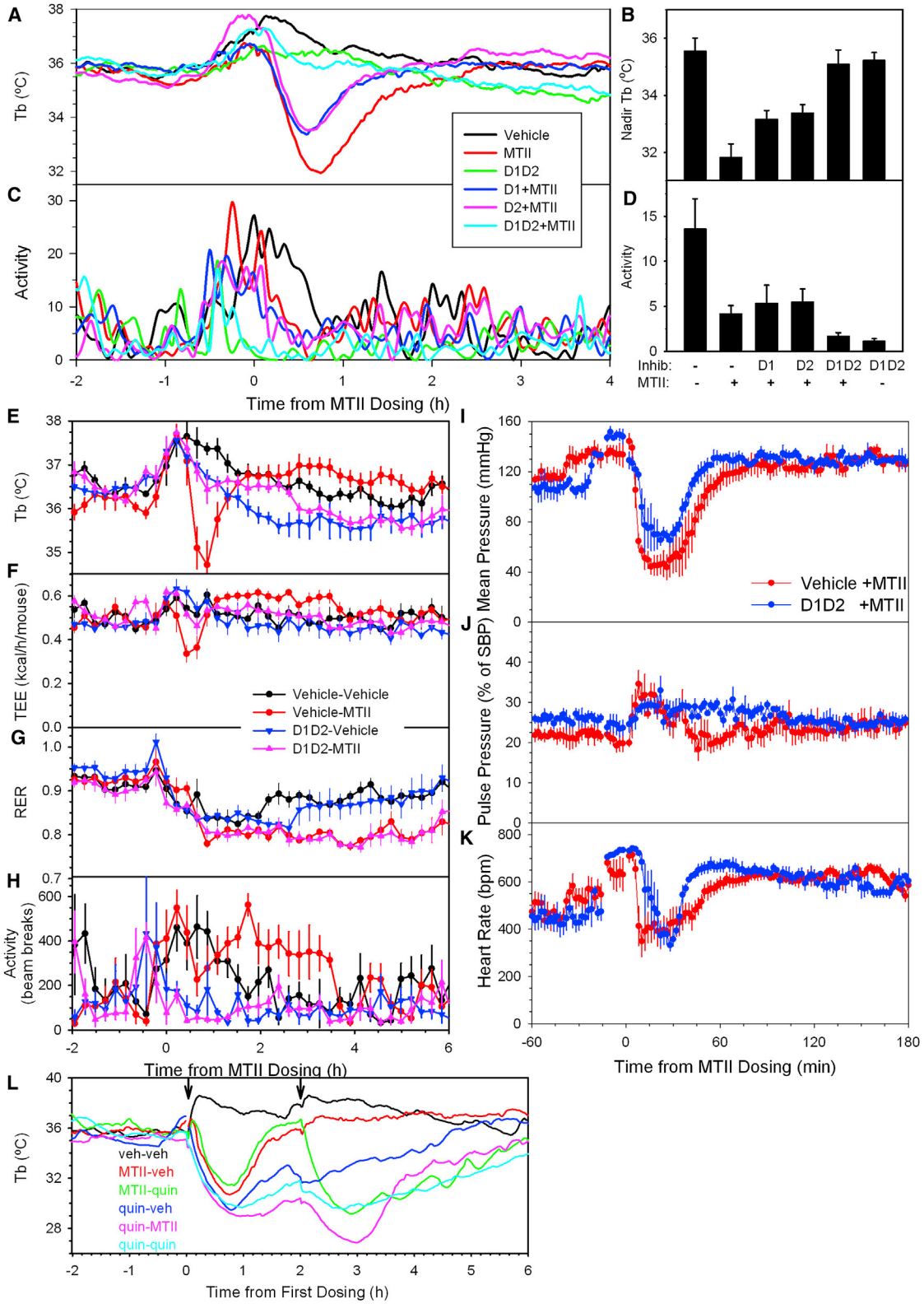

Figure 6. Role of Dopamine in Mediating the Hypothermic Response to MTII

(A–D) We measured Tb (A), nadir Tb (B), activity (C), and mean activity (mean of 10–60 min) (D) in chow-fed mice (30.0 ± 0.3 g) at 22°C pretreated with vehicle, D1 antagonist (SCH23390, 2 mg/kg), or D2 antagonist (sulpiride, 30 mg/kg) 15 minutes before treatment with vehicle or MTII (n = 7–8/group). Activity is in arbitrary units via Mini Mitter.

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Our working model (Figure 7D) is that MTII causes hypometabolism/hypothermia via dopamine neurons, possibly in the arcuate nucleus. The dopamine acts on D1 and D2 receptors to lower Tb. It is not known if MTII is acting directly on the dopaminergic neurons or on more upstream neurons. We focused on dopamine because MTII hypothermia is inhibited by dopamine antagonists, but not by other inhibitors of pharmacologic hypothermia (antagonists of mu, kappa, and delta opioid, 5HT1A, and cannabinoid-1 receptors). Dopamine regulates Tb in the preoptic area/anterior hypothalamus (Boulay et al., 1999; Brown et al., 1982; Cox and Lee, 1977). It is possible that the arcuate nucleus dopaminergic neurons that are still activated by MTII in *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ mice are part of the circuit between MTII-responding cells and the hypometabolism/hypothermia.

The physical site of MTII action causing hypometabolism/hypothermia is not known (Warne and Xu, 2013). Since hypermetabolism is a centrally mediated effect, the similar ED_{50}s for hyperthermia and hypothermia are consistent with hypometabolism/hypothermia also being a central effect. In rabbits, melanocortin injection into the preoptic and septal regions (which regulate Tb; Nakamura, 2011) inhibited fever (Feng et al., 1987; Glyn-Ballinger et al., 1983). However, a more anatomically diverse regulation of MTII effects has been proposed since local MTII injections into the nucleus tractus solitarius, paraventricular nucleus, rostral ventrolateral medulla, parabrachial nucleus, and the retrochiasmatic area all increased Tb (Skibicka and Grill, 2009). Mc4r reactivation in paraventricular nucleus (PVN) neurons restores the food intake response to MTII, but not the metabolic rate effects (Balthasar et al., 2005). Metabolic rate and blood pressure effects of MC4R are mediated by cholinergic preganglionic neurons in the dorsal motor nucleus of the vagus and intermediolateral column (Rossi et al., 2011; Sohn et al., 2013).

After its rapid onset, the hypothermic/hypometabolic state is self-limited, being largely reversed by ~1 hr and refractory to a second dose of MTII. These kinetics are not a simple function of MTII pharmacokinetics since the Tb changes are independent of MTII dose over a 4-fold range and thus likely independent of plasma MTII levels (MTII plasma elimination t½ is ~20 min; Hatziieremia et al., 2007). The intact rewarming in *Ucp1*$^{-/-}$ mice demonstrates that the backup mechanisms invoked to maintain Tb in these mutant mice (Bal et al., 2012; Liu et al., 2003) are also sufficient for rewarming after MTII hypothermia. These data suggest that the hypothermic/hypometabolic response is a triggered process of limited duration that is followed by a refractory period; the hypermetabolism does not exhibit a refractory period.

What is the physiologic significance of the hypometabolism/hypothermia? All five of the melanocortin receptors have anti-inflammatory activities (Caruso et al., 2007; Catania et al., 2004, 2010; Getting et al., 2008; Lee and Taylor, 2013; Li and Taylor, 2008). It seems plausible that the hypometabolism/hypothermia/hypotension effects are part of a physiologic response to limit stress/inflammation and increase survival. Indeed, melanocortin agonism increases survival in a mouse model of multiple organ dysfunction (Bitto et al., 2011). In humans, hypothermia is endogenously generated in ominous clinical situations, including severe trauma (Shafi et al., 2005) and sepsis (Clemmer et al., 1992). Hypothermia/hypometabolism is also induced therapeutically to improve clinical outcomes, such as with hypoperfusion surgery (Lampe and Becker, 2011) and after hypoxic/hypoperfusion injury (Holzer, 2010). We hypothesize that hypometabolism/hypothermia is a conserved, organized, and regulated physiologic response to dire situations, and not dysregulated thermal regulation. Just as fever increases the inflammatory response to fight infection (Mackowiak, 1998), hypothermia puts a brake on it, with beneficial effect in some situations.

Melanocortin hypermetabolism/hyperthermia is distinguished from the hypometabolism/hypothermia by its timing, lack of attenuation, and selectivity for MC4R. The metabolism/obesity field has focused on the hypermetabolism/hyperthermia, as this melanocortin physiology is relevant for understanding food intake, metabolic rate, body weight and adiposity, and insulin sensitivity. Melanocortin agonism is a potential treatment for obesity but has been hampered by autonomic effects, including increased blood pressure (Greenfield et al., 2009). Our observations suggest that there may be other effects of high doses of such drugs. The hypometabolic/hypothermic and hypermetabolic/hyperthermic responses are distinct physiologic processes with distinguishable drivers and biologic effects. Further understanding of the hypometabolic/hypothermic response could lead to better understanding of when to use clinical hypothermia, improved methods for inducing, maintaining, and reversing clinical hypothermia, and optimized treatment of accidental hypothermia.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 male mice (Jackson Laboratory) were singly housed at ~22°C, fed NIH-07 chow (15% kcal from fat, Harlan Laboratories) or D12492 (60% kcal from fat, Research Diets), and studied between 14 and 37 weeks of age. Mice were maintained at 21°C–22°C with lights on 6 a.m.–6 p.m. Food and water were available ad libitum, including during drug treatments and indirect calorimetry. All animal studies were approved by the NIDDK/NIH Animal Care and Use Committee. *Mc4r*$^{-/-}$ mice (loxTB *Mc4r*; *Mc4r*^{tm1Lowl}/J) (Balthasar et al., 2005), *Mc3r*$^{-/-}$ mice (loxTB *Mc3r*; *Mc3r*^{tmButl}/J) (Begriche et al., 2011), and *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ mice were generated by heterozygote mating and studied on mixed genetic backgrounds using littermates as controls. *Mc1r*$^{e/e}$ mice (81% C57BL/6J) (Robbins et al., 1993) were provided by Dr. David Fisher (MGH). *Mc5r*$^{-/-}$ mice (Chen et al., 1997) were supplied by Dr. Andrew Taylor (Boston University Medical Center, C57BL/6J background). *A*^{vy}/+ mice (B6.C3-*A*^{vy}/J) (Yen et al., 1994) and *Ucp1*$^{-/-}$ (B6.129-Ucp1^{tm1Kz}/J) (Enerbäck et al., 1997) were purchased from Jackson Laboratory. Mice were studied ≥7 days after any operation or prior treatment. Reuse of mice tends to reduce physical activity levels, presumably due to acclimatization. No specific effort was made to acclimatize mice to handling in individual experiments.

(E–H) We measured Tb (E), TEE (F), RER (G), and activity (H) at 22°C in chow-fed mice (27.2 ± 0.3 g) pretreated with vehicle, D1and D2 antagonists (SCH23390, 2 mg/kg and sulpiride, 30 mg/kg) 15 minutes before treatment with vehicle or MTII (n = 6/group).
(I–K) We measured mean arterial pressure (I), pulse pressure (J), and heart rate (K) in ambulating telemetered mice at 22°C pretreated with vehicle or D1 and D2 antagonists (SCH23390, 2 mg/kg i.p. and sulpiride, 30 mg/kg i.p.) 15 minutes before treatment with MTII at t = 0 in a crossover design (n = 3–4/group).
(L) Chow-fed mice (29.2 ± 0.3 g) at 22°C were treated with vehicle, MTII, or quinpirole at 0 hr and again 2 hr later, as indicated (n = 4/group). Data are mean ± SEM.

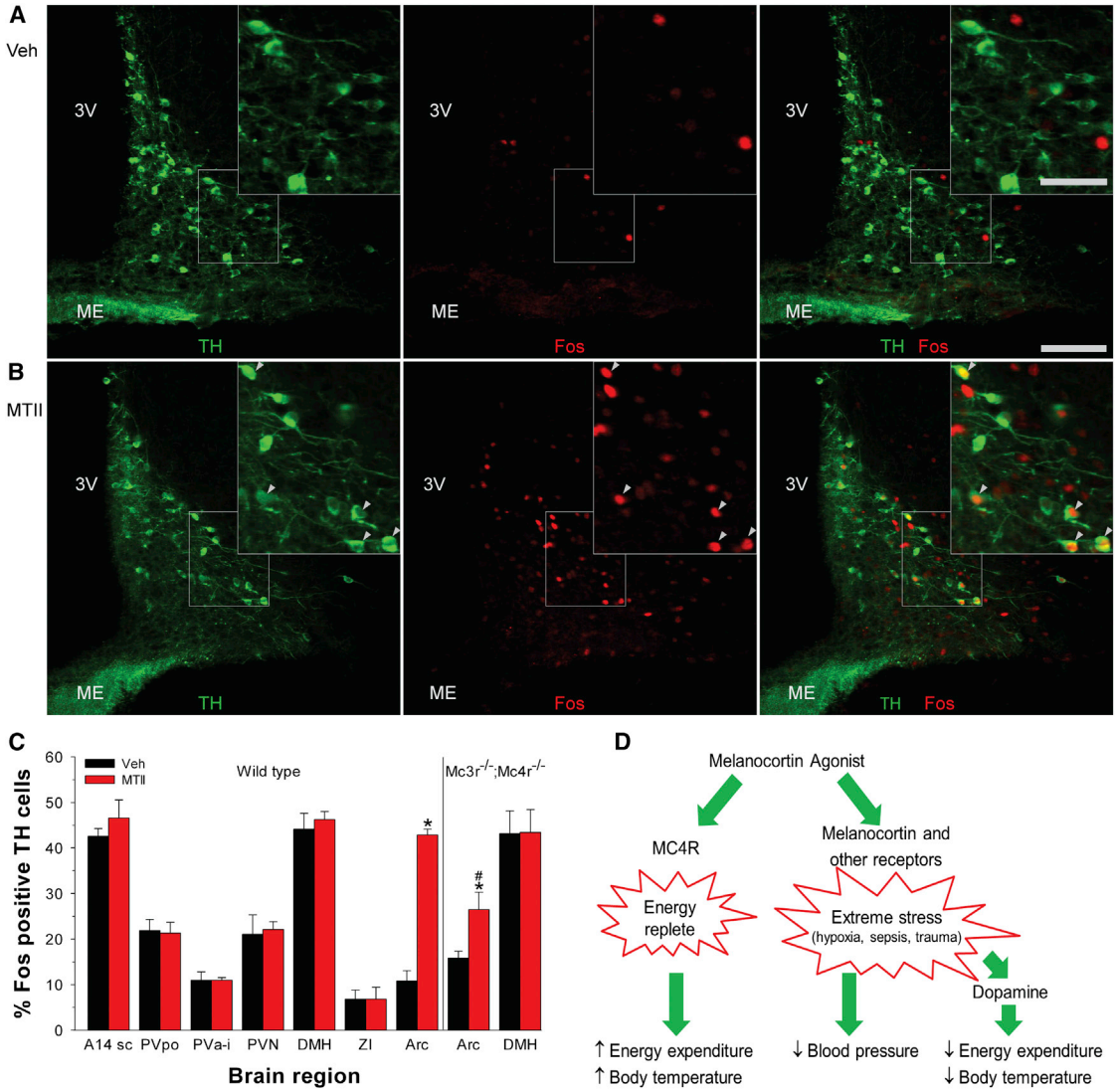

Figure 7. MTII Activates Dopaminergic Neurons Selectively in the Arcuate Nucleus

(A and B) Immunohistochemistry for TH (green; left panel) and Fos (red; middle panel) after vehicle (A) or MTII (B) treatment of C57BL/6J mice. Scale bar represents 100 μm in main image and 50 μm in insert. Arrowheads indicate Fos-positive TH neurons; 3V, third ventricle; ME, median eminence.

(C) Percentage of TH-immunoreactive neurons staining for Fos after vehicle or MTII administration in wild-type (n = 3/group) and *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$ mice (n = 4/group). Neurons were counted in the subcommissural part of the A14 dopaminergic cell group (A14sc; 2–3 sections), preoptic part of the periventricular hypothalamic nucleus (PVpo; 3 sections), paraventricular nucleus of the hypothalamus (PVN; 6–7 sections), anterior and intermediate part of the periventricular hypothalamic nucleus (PVa-i; 6–7 sections), dorsomedial hypothalamic nucleus (DMH; 2–3 sections), zona incerta (ZI; 3 sections), and arcuate nucleus (Arc; 6–7 sections). The number of TH neurons per region was not statistically different between the MTII and vehicle groups, and the analyzed areas had expected distributions of TH-immunoreactive neurons (Lookingland and Moore, 2005). Data are mean ± SEM; *p < 0.05 versus vehicle. #p < 0.05, wild-type versus *Mc3r*$^{-/-}$;*Mc4r*$^{-/-}$.

(D) Actions of melanocortin agonists. Melanocortin agonists act via Mc4r to signal that the body is in an "energy replete" state, detectable for example as an increase in energy expenditure and Tb during fasting. Melanocortin agonists also act likely via both melanocortin and other receptors to signal "extreme stress" caused by factors such as hypoxia, sepsis, and severe trauma. This triggers a time-limited set of responses, including hypometabolism and hypothermia mediated by dopaminergic pathways and also hypotension, which appears not to occur via dopaminergic pathways.

Drugs

Drugs (Bachem for MTII; otherwise Tocris or Sigma) were administered at 10–11 a.m. with the indicated dosing concentration, dose, route, and vehicle: MTII (up to 2 mg/ml i.p., saline; dosed at 10 mg/kg i.p. when not otherwise specified), NDP-MSH (100 and 400 μg/mouse i.p., saline), sulpiride (3 mg/ml, 30 mg/kg i.p., 100% DMSO, heated to 60°C, then 9 vol of saline), SCH23390 (0.4 mg/ml, 2 mg/kg i.p., saline), quinpirole (0.1 mg/ml, 1 mg/kg i.p., water), AM251 (1 mg/ml, 10 mg/kg per os, 100% DMSO, heated to 60°C, then 1 vol Tween 80 and 8 vol of saline), naloxone (1 mg/ml, 10 mg/kg i.p., saline), naltrindole (0.5 mg/ml, 5 mg/kg i.p., saline), and WAY100635 (0.1 mg/ml, 1 mg/kg s.c., water).

Tb Telemetry

Tb and activity were continuously measured by telemetry (Mini Mitter/Philips Respironics) using ER4000 energizer/receivers, G2 E-mitters implanted intraperitoneally, and VitalView software with data collected each minute. The

hypothermia metric is the nadir of Tb at 20–50 min after melanocortin dosing. The hyperthermia metric is the mean Tb at 120–300 min minus the mean Tb at −150 to −30 min relative to dosing. ED_{50}s were calculated by fitting to a four-parameter logistic curve using SigmaPlot v.12.5. BAT temperature and Tb were measured simultaneously in mice carrying two IPTT-300 transponders (Bio Medic Data Systems), one sutured to the omentum and the other sutured underneath the interscapular BAT.

Blood Pressure Telemetry

Chronic ambulatory arterial blood pressure and heart rate were measured with radio transmitters (model TA11PA-C10; Data Sciences International) implanted during ketamine and xylazine anesthesia in the carotid artery as described (Oppermann et al., 2009). Data were sampled for 10 s every 2 min and processed using a model RPC-1 receiver, a 20-channel data exchange matrix, APR-1 ambient pressure monitor, and a Data Quest ART Silver 2.3 acquisition system.

Temperature Preference Test

Mice were placed in a stainless steel pan (64 × 15 × 20 cm, length × width × height) spanning two hot/cold plates such that a temperature gradient from 18°C to 36°C was established across the floor of the pan. Mice were placed into this pan for 90 min, and their position was tracked with an overhead camera and video tracking software (EthoVision 9.0, Noldus). The temperature gradient was calibrated with a FLIR T400 infrared camera, and these images were used to transform the position data into temperature.

Calorimetry

An Oxymax/CLAMS (Columbus Instruments) was used to measure Tb, energy expenditure (O_2 consumption and CO_2 production), and activity by beam break simultaneously in mice implanted with G2 E-mitters. RER is the ratio of CO_2 produced to O_2 consumed. Experiments were performed at 22°C or 30°C, as indicated. Sampling was typically every 13 minutes, measuring from 12 chambers. When better time resolution was required, only six chambers were studied, allowing sampling every 6 minutes.

Immunohistochemistry

Male mice were housed individually from day −7 and, during the 4 days preceding the experiment, habituated to handling and i.p. injection. On the day of the experiment, mice received MTII (10 mg/kg, i.p.) or vehicle, returned to their home cage and were anesthetized 60 minutes later, had their rectal temperature measured, and were perfused with 10% formalin solution. Brains were fixed for 2 hr, transferred to 30% sucrose solution for 2 days, frozen, and sectioned (Leica SM2010R). A 1-in-3 series of coronal 40 μm sections was incubated (overnight, room temperature, constant agitation) with antibodies to TH (Millipore, MAB318; 1:1,000 dilution) and Fos (Calbiochem, PC38; 1:7,000 dilution). After rinsing, the sections were incubated in Alexa Fluor 488 anti-mouse and Alexa Fluor 555 anti-rabbit (1:500 dilution) for 2 hours, rinsed, and mounted using ProLong Gold Antifade medium. Images were captured (Olympus VS120 Slide Scanner microscope) and analyzed with OlyVIA software (v.2.6) or with a Zeiss LSM 510 confocal microscope. A neuron was scored TH-immunoreactive only if its nucleus was visible and surrounded by a rim of TH immunofluorescence. Anatomy was defined according to (Franklin and Paxinos, 2008), with periventricular hypothalamic nucleus subdivisions per Allen Mouse Brain Atlas (http://mouse.brain-map.org/static/atlas). We did not use a stereological procedure or counting correction factor since we are testing for changes between genotype and/or treatment. The percentage of MTII treatment-activated TH neurons is the number of Fos-positive TH-immunoreactive neurons divided by the total number of TH-immunoreactive neurons.

Statistics

Data are reported as mean ± SEM. Significance (two-tailed $p < 0.05$) was determined using SigmaPlot using t test or two-way ANOVA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.05.021.

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